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EFFICIENT TRANSLATION IN CHLOROPLASTS REQUIRES ELEMENT(S) UPSTREAM OF THE PUTATIVE RIBOSOME BINDING SITE FROM *ATPI*[1]

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Thousands of proteins make up a chloroplast, but fewer than 100 are encoded by the chloroplast genome. Despite this low number, expression of chloroplast-encoded genes is essential for plant survival. Every chloroplast has its own gene expression system with a major regulatory point at the initiation of protein synthesis (translation). In chloroplasts, most protein-encoding genes contain elements resembling the ribosome binding sites (RBS) found in prokaryotes. In vitro, these putative chloroplast ribosome binding sequences vary in their ability to support translation. Here we report results from an investigation into effects of the predicted RBS for the tobacco chloroplast *atpI* gene on translation in vivo. Two reporter constructs, differing only in their 5′-untranslated regions (5′UTRs) were stably incorporated into tobacco chloroplast genomes and their expression analyzed. One 5′UTR was derived from the wild-type (WT) *atpI* gene. The second, Holo-substitution (Holo-sub), had nonchloroplast sequence replacing all wild-type nucleotides, except for the putative RBS. The abundance of reporter RNA was the same for both 5′UTRs. However, translation controlled by Holo-sub was less than 4% that controlled by WT. These in vivo experiments support the idea that translation initiation in land plant chloroplasts depends on 5′UTR elements outside the putative RBS.

Key words: chloroplast; gene expression; land plant; ribosome binding site; translation; untranslated region.

Plastids are descended from prokaryotic endosymbionts, and chloroplast gene expression still has many of the features of its prokaryote ancestors (Sugita and Sugiura, 1996; Esposito et al., 2003; Yu et al., 2005; Bohne et al., 2006; López-Juez, 2007). Plastids contain their own genomic DNA, separate from that in the nucleus. However, during the course of evolution, many plastid genes have been exported to the nucleus, while other nucleus-encoded genes have acquired novel functions in the plastid and/or displaced plastid-encoded genes in the chloroplast (Yamaguchi et al., 2003; Stegemann and Bock, 2006; Raynaud et al., 2007; Deschamps et al., 2008). The acquisition of nonprokaryotic elements to control gene expression has greatly increased the complexity of gene expression in plastids (reviewed in Barkan and Goldschmidt-Clermont [2000]; Marín-Navarro et al. [2007]).

Proper regulation of chloroplast gene expression in response to environmental and developmental cues is crucial for plant survival. Regulation of chloroplast gene expression can be broadly divided into two processes. The first is transcription, which determines whether (and when) a chloroplast gene is used as a template for the production of RNA. The second broad regulatory category is posttranscriptional. Although transcription is the fundamental first step of gene expression, it has been shown that chloroplast protein accumulation can vary over 10 000-fold for genes with the same promoter (transcriptional control region) (Maliga, 2003). Thus posttranscriptional mechanisms are the primary regulators of chloroplast gene expression (Monde et al., 2000; Nakamura et al., 2004; Rochaix, 2006; Marín-Navarro et al., 2007). Chloroplast mRNA functionality can be altered by physical modification of the RNA (editing, splicing, cleavage) and/or RNA stability (Monde et al., 2000; Schuster and Bock, 2001; Asakura and Barkan, 2006; Hayes and Hanson, 2007; Yukawa et al., 2007). Translation is particularly strongly regulated in the chloroplast, at both initiation and elongation levels (reviewed in Sugiura et al. [1998]; Zerges [2000]; Marín-Navarro et al. [2007]).

In prokaryotes, almost every mRNA leader contains a ribosome binding site (RBS) that greatly enhances translation initiation (McCarthy, 1994; Gualerzi et al., 2000). The RBS contains a Shine–Dalgarno (SD) sequence that is located ~7 nt upstream of the start codon in the leader of *E. coli* mRNAs (Shine and Dalgarno, 1974). The 3′ end of the 16S rRNA in the 30S small subunit of the prokaryotic ribosome hybridizes to the SD sequence, which results in translation initiation at the correct site (Shine and Dalgarno, 1974).

In eukaryotes, the mRNA leader region, which is all the nucleotides prior to the translation start site, is called the 5′-untranslated region (5′UTR). Shine-Dalgarno-like (SD-like) sequences are located within the 5′UTR near translation start sites in approximately two-thirds of plastid genes (reviewed in Bonham-Smith and Bourque [1989]; Hirose and Sugiura [2004]). Unlike in prokaryotes, the composition and the position of SD-like sequences in plastid genes are not conserved. The putative ribosome binding sites on some chloroplast mRNAs function in translation in vitro, while others do not (Betts and Spremulli, 1994; Fargo et al., 1998; Plader and Sugiura, 2003; Hirose and Sugiura, 2004). Therefore, it appears that chloroplasts may have evolved an SD-sequence-independent mechanism of translation initiation for many of its mRNAs. The current hypothesis is that to be functional, a putative plastid RBS must be located around 10 nt upstream of the translation

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start site and be composed of SD-like sequence elements (some derivative of GGAGG) (Hirose and Sugiura, 2004).

Putative ribosome binding sites have also been found to have a variety of effects on translation in vivo. In tobacco chloroplasts, Eibl and colleagues (1999) found that a truncated 5′UTR that possessed a putative RBS was actually more efficient at translation than the full-length 5′UTR. In contrast, Sakamoto and colleagues (1994) found that mutations to a putative RBS had no effect on expression of a reporter gene in chloroplasts of the green alga *Chlamydomonas reinhardtii*.

Here we report results of an in vivo study to analyze the role of a putative RBS in a 5′UTR of wild-type length in tobacco chloroplasts. To simplify the analysis, we used a single representative chloroplast 5′UTR as the benchmark for all our experiments. It is derived from the *atpI* gene, which encodes the CF_o-IV subunit of the ATP synthase complex (Hudson and Mason, 1988; Stollar and Hollingsworth, 1994; Miyagi et al., 1998). Two reporter gene constructs were created to test whether the putative RBS from *atpI* could mediate translation in vivo. One encoded the wild-type 5′UTR, while the second had a full-length 5′UTR that was, except for the putative RBS, nonchloroplast sequence. We discovered that even though the SD-like sequence in the *atpI* 5′UTR contains features that are hypothesized to be sufficient for a functional RBS, it only supports very inefficient translation in the absence of upstream chloroplast-derived sequences in vivo.

MATERIALS AND METHODS

Plants—*Nicotiana tabacum* var. Petit Havana were used for all experiments. Seedlings for transformation were grown from 16 seeds evenly spaced within a 3-cm diameter circle on Murashige-Skoog basal agar media, with 3% sucrose (Murashige and Skoog, 1962). The seedlings were grown for 15 d. Plants for gene expression assays were grown in soil. Seedlings and plants were grown under ~100 μmol·m^{-2}·s^{-1} fluorescent light, with a regimen of 16-h days and 8-h nights at a temperature of 23°C.

In vitro reactions for generating DNA fragments and RNA probes—Primers and templates for these reactions are listed in Table 1.

Polymerase chain reaction (PCR) conditions were according to supplier protocols, with 10 ng/μL template, 0.6 pm/μL of each primer, and 0.05 U/μL *Taq* polymerase (New England Biolabs [NEB], Beverly, Massachusetts, USA). Hybridization temperatures were set at T_m − 10°C, and there were 30 cycles. The T_m was calculated using OligoAnalyzer version 3.1 (XX-IDT Sci Tools, website http://www.idtdna.com).

Small DNA fragments were produced using a fill-in reaction with the Klenow subunit of *E. coli* DNA polymerase I, according to supplier's protocols (NEB). Reactions contained 0.24 pm/μL of both oligonucleotides, 0.2 mM of each dNTP, and 0.05 U/μL Klenow.

Probes for Southern blots and ribonuclease protection assays (RPAs) were generated by in vitro transcription. Transcription reaction conditions were according to supplier's protocols. Reactions to transcribe radiolabeled probes contained 5 pm/μL template (PCR fragment), 0.5 mM of all four NTPs, and 20 U of T7 RNA polymerase (NEB) in a total of 20 μL. Transcription reactions to generate 16S and *uidA* probes also contained 120 μCi α-^{32}P-UTP, while *rbcL* reaction mixtures included 1.2 μCi (3000Ci/mmole EasyTide, Perkin-Elmer, Waltham, Massachusetts, USA). After transcription, the *rbcL* and *uidA* reactions were incubated with 10 U DNase I according to supplier's protocols to digest the DNA template (NEB).

Gel purification of probes—RNA probes used in RPAs were subjected to electrophoresis in 5% acrylamide (29 : 1 acrylamide : bis-acrylamide)–7 M urea gels. Gel fragments containing the RNAs were crushed in diethylpyrocarbonate (DEPC)-treated 0.3 M NaCl and placed on a rocking platform overnight at 4°C. The solution was passed through a 0.22-μm, syringe-driven filter (Millex GP, Millipore, Bedford, Massachusetts). RNA in the filtrate was precipitated with ethanol. RNA concentration was quantified by scintillation counting.

Construction of plasmids for chloroplast transformation—A reporter gene with unique HindIII and EcoRI restriction sites on the 5′ and 3′ ends, respectively, was ligated into a HindIII-/EcoRI-digested PRV111B transformation vector (Zoubenko et al., 1994). The reporter gene consisted of four sections: the native tobacco chloroplast *atpI* transcription promoter, some variant of the *atpI* 5′UTR, an open reading frame (ORF) encoding *uidA*, and the 3′UTR from the tobacco

TABLE 1. Primers and templates for construction of clones and transcription/sequencing templates. (Restriction sites are underlined)

Template	Primers (forward/reverse)	Reaction	Restriction sites (5′/3′)	Product	Purpose
—	GCAAGCTTCTGAATTTCAAAAAAGAGATAAAA/ CCGCTGCAGCCCCAGCTATTTTTATCTCTTT	Klenow fill-in	HindIII/PstI	*atpI* promoter	cloning
pBI221 (Clontech)	CCATGGCCATGGTACGTCCTGTAGAAACCCCAA/ GCGAGCTCGGTAGCAATTCCCGAGGCTGT	PCR	NcoI/SacI	*uidA* ORF	cloning
Total tobacco leaf DNA	GCCTGCAGATATTATGTGATTT / CCATGGCCATGGTGCCTTGCCCTC	PCR	PstI/NcoI	Wild-type *atpI* 5′UTR	cloning
Total tobacco leaf DNA	CGGAGCTCGAGAAATTCAATTAAGGAAATAA / CGGAATTCAATGGAA	PCR	SacI/EcoRI	*rps16* 3′UTR	cloning
pBI221 (Clontech)	CCGCTGCAGATTTCTCAAGATCAGAAGTA / CCATGGCCATGGTGCCTTGCCCTCCCTTTGAT TCAGTGG	PCR	PstI/NcoI	Holo-sub 5′UTR	cloning
Total tobacco leaf DNA	TTCGACCCTGATTATCCC / TAATCGACTCACTATAGGGTAAGCTATTGCCT CACC	PCR		16S template	Southern probe
Total tobacco leaf DNA	GAGCGCCAGTCGTCGTCGGCTCTCGATGTCAC CACAAACAGAGACTAAAGC / TAATACGACTCACTATAGGGCCTGAAGCTGCG GGCATTCCCGATCGTCCATGTACCAGTAGAAG ATTCGG	PCR		*rbcL* probe template	RPA probe
Transformation plasmid with *uidA* gene	AGTGCCAGTAAAGCGCCGGCTGCTGATGTTA CGTCCTGTAGAAACCCCA / TAATACGACTCACTATAGGGTCGAGCAGCGC CCGCTTGTTCCTGAATTGACCCACACTTTGCC GTAATGA	PCR		*uidA* probe template	RPA probe
Total tobacco leaf DNA	ATGGAAGGCTTTTTATTCAACAGTATAACA / TCTCTGAATGCCCACAGGC	PCR		promoter + 5′UTR	sequencing template

chloroplast *rps16* gene (Fig. 1, see *Gene abbreviations* section for explanations of gene names). Fragments for cloning were created from templates and primers as listed in Table 1. The Holo-substitution (Holo-sub) transformation plasmid differs from the wild-type (WT) plasmid only in the 5′UTR (as noted); identical bases at the 5′ end, which include the transcription start site (GGGG) and a cloning site (PstI), are underlined, and the putative RBS is in bold face:
WT 5′UTR—GGGGCTGCAGATATTATGTGATTTATTAGTATTCTAAA TCTTAGTTGGTATTCAAAATATCCGATTCAAGTAGACAAAGAGAT GGTTGAATCAAAAAATTTTGTTTAAAGTTCAATTTTTTCA**GAGGG-CAAGGCACC**; Holo-sub 5′UTR—GGGGCTGCAGATTTCTCAAGATCA GAAGTACTATTCCAGTATGGACGATTCAAGGCTTGCTTCACAAACA AGGCAAGTAATAGAGATTGGAGTCTCTAAAAAGGTAGTTCCCACT-GAACAAAGG**GAGGGCAAGGCACC.**

Chloroplast transformation—Biolistic transformation was performed essentially as described by Bock (1998). DNA-coated tungsten pellets were forced through the leaf adaxial face of 15-d-old plantlets using an HE-1000 Biolistic Gene Gun (Bio-Rad, Hercules, California, USA). Transformed plants were selected and regenerated as by Svab and colleagues (1990). When the rooted transformed plantlet had 4–6 leaves, total DNA was isolated from one leaf and examined by Southern analysis using a probe complementary to the transformation target region (see *Verification of transformants*) (Southern, 1989). Plants homoplasmic for the transformation construct were transplanted to soil, self-fertilized, and grown to seed. Gene expression assays were performed with plants that were from the T2 or later generation.

Verification of transformants—Transformation plasmids were targeted to recombine into a transcriptionally silent region between the *trnV* and 3′*rps12* genes in the inverted repeat (IR) region of the chloroplast genome (Fig. 1). To test whether the plasmid successfully recombined into the targeted region, 0.1 g of leaf material was crushed in liquid nitrogen and the total DNA of the leaf extracted using a Qiagen DNEasy Plant Mini kit (Qiagen, Valencia, California, USA). Five micrograms of total DNA was digested with 50 U each of EcoRI and EcoRV as per supplier's instructions (NEB). DNA isolated from untransformed tobacco was used as the negative control. Restricted DNA fragments were subjected to Southern analysis using supplier's protocols (Nytran SuperCharge, Whatman, Florham Park, New Jersey, USA) (Southern, 1989).

A uniformly radiolabeled RNA complementary to the tobacco plastid 16S DNA region surrounding the transformation target site was used as probe (see Table 1 for probe templates). Results were visualized using the Storm 820 phosphorimager (GE Lifesciences, Piscataway, New Jersey, USA). A correctly targeted reporter construct resulted in an EcoRI-EcoRV fragment that was 1 kb larger than the untransformed EcoRI-EcoRV fragment (untransformed = 2.4 kbp, transformed = 3.4 kbp).

To ensure that the variable 5′UTR was correct, a fragment containing the promoter and 5′UTR region from each unique transformant was amplified by PCR and sequenced (data not shown). Only those transformants with the correct promoter/5′UTR sequence were used for further experiments.

Isolation of intact tobacco chloroplasts—Isolation of chloroplasts from 15 g tobacco leaves (8–10 cm) was performed as in Yukawa et al. (2007) with minor modifications. Intact chloroplasts were collected from the 45%/80% in-

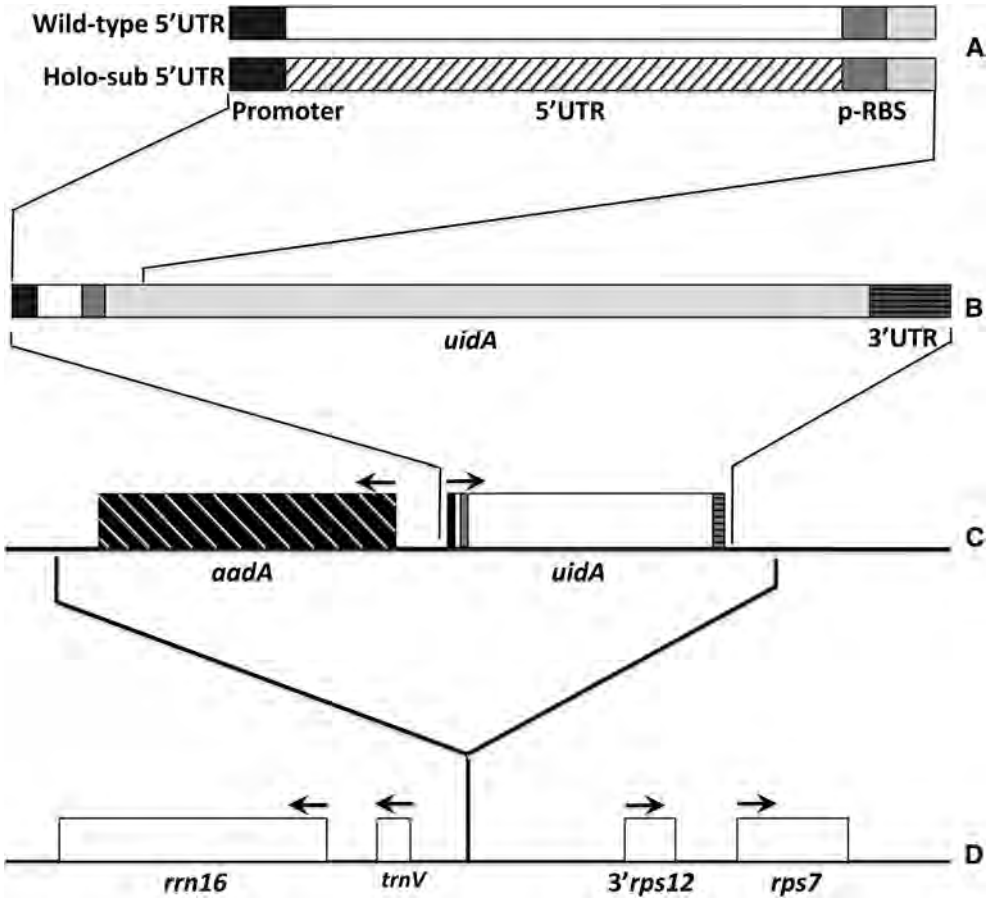


Fig. 1. Diagram of reporter gene constructs and transformation target. (A) Diagram of the 5′-untranslated region (5′UTR) used in these experiments. Black boxes designate the consensus promoter from tobacco chloroplast *atpI*. The white box denotes wild-type chloroplast sequence; the black-ribbed box denotes nonchloroplast sequence; "p-RBS" is the putative RBS from tobacco chloroplast *atpI*. (B) The entire reporter construct consists of four sections: the promoter, the 5′UTR, the open reading frame from *uidA*, and the 3′UTR from tobacco chloroplast *rps16*. (C) Reporter genes were incorporated into the transformation vector pPRV111B, which contains the antibiotic resistance gene *aadA*. Arrows represent the direction of transcription. (D) The region of the tobacco chloroplast genome to which the transformation cassette is targeted. (Gene abbreviations are defined in Materials and Methods)

terface of a Percoll step-gradient (GE Lifesciences , Piscataway, New Jersey, USA). After concentration, chloroplasts were resuspended in 2 mL TE buffer (10 mM Tris pH 7.5, 1 mM EDTA) with 2.5 mM dithiothreitol (DTT) and either frozen at −70°C or used immediately.

Isolation of total chloroplast RNA—Intact tobacco chloroplasts from 15 g leaf tissue were extracted six times with equal volumes of phenol and chloroform : isoamyl alcohol (24 : 1). After the last extraction, the nucleic acids were concentrated by ethanol precipitation and resuspended in DEPC-treated water. Resuspended nucleic acids were treated with RNase-free DNase I using the supplier's protocol (NEB).

Ribonuclease protection assays—Reporter RNA abundance was determined by RPAs. Assays were performed according to the RPA III kit protocol (with 1 : 100 RNase dilution; Ambion, Austin, Texas, USA). Each probe had 25 nt of nonchloroplast sequence flanking both sides of its target sequence, to provide an internal control for RNase digestion. After digestion, samples were subjected to electrophoresis in a 5% acrylamide (29 : 1 acrylamide : bis)/7 M urea gel. Each gel was dried onto 3MM paper (Whatman), imaged using a Storm 820 and analyzed using ImageQuant (GE Lifesciences) and Microsoft (Redmond, Washington, USA) Excel software. Each set of assays was performed with identical concentrations of radiolabeled probe and four concentrations of tobacco chloroplast RNA. Only those sets of reactions in which the signal changed in proportion to the quantity of chloroplast RNA, demonstrating that the probe concentration was in excess over the concentration of target RNA, were used in further analysis.

β-glucuronidase assays—The reporter gene in these transformants, *uidA*, encodes β-glucuronidase. β-Glucuronidase activity was assayed to quantify reporter mRNA translation (Jefferson, 1987). Plant cell extracts were isolated from six 6-mm diameter leaf sections, taken from evenly spaced positions along ~8 cm long leaves. Protein concentration was determined with a Bradford assay (Sigma, St. Louis, Missouri, USA). β-Glucuronidase was assayed by hydrolyzing the glucuronide moiety from methylumbelliferone glucuronide (Jefferson, 1987). The product, methylumbelliferone (MU) was detected with a Synergy HT (BioTek, Winooski, Vermont, USA) fluorimeter (excitation λ = 360 nm, emission λ = 460 nm). A 10 pmol–750 pmol standard curve was used to calculate the concentration of MU in each sample. Specific activity was calculated as picomoles of MU produced per minute per microgram of total protein.

Gene abbreviations—The following genes are mentioned in the text: *aadA*, prokaryotic streptomycin/spectinomycin adenylyltransferase; *atpE*, chloroplast-encoded epsilon subunit of CF_1 of the H^+-translocating ATP synthase; *atpI*, chloroplast-encoded subunit IV of CF_o of the H^+-translocating ATP synthase; *petB*, chloroplast-encoded cytochrome b_6; *psbA*, chloroplast-encoded D1 protein from photosystem II; *rbcL*, chloroplast-encoded large subunit of ribulose bis-phosphate carboxylase; *rps2*, chloroplast-encoded small ribosomal protein subunit 2; *rps7*, chloroplast-encoded small ribosomal protein subunit 7; *rps12*, chloroplast-encoded small ribosomal protein subunit 12; *rps16*, chloroplast-encoded small ribosomal protein subunit 16; *rrn16*, chloroplast-encoded 16S rRNA; *trnV*, chloroplast-encoded tRNA-valine; *uidA*, prokaryotic β-glucuronidase.

RESULTS

Two 5′UTR variants were used to analyze RBS function in vivo—A pair of reporter gene constructs differing only in their 5′UTR sequences was created to test the role of a putative RBS in expression of a reporter gene in vivo (Fig. 1). The wild-type (WT) 5′UTR construct has 125 nt of the 131 nt wild-type *atpI* 5′UTR, including the putative RBS. The Holo-substitution (Holo-sub) 5′UTR derivative consists of the putative ribosome binding site from the *atpI* 5′UTR (−1 to −14) and 112 nt (−15 to −126) of sequence derived from the cauliflower mosaic virus 35S promoter. The constructs were transformed into the transcriptionally silent region between the *3′rps12* and *trnV* genes of the tobacco plastid genome (Fig. 1 and [Maliga, 2004]).

Analyzing transplastomic plants for insertion location and homoplasmy—Every chloroplast has between 10 and 100 copies of its genome. To ensure that a transformed genome can be propagated without selective pressure, the chloroplast must become homoplasmic (i.e., all genome copies identical) for the transformed genome during selection. Homoplasmy also alleviates any gene dosage effects that might occur in later assays if different transformed plants varied in the proportion of transformed genomes that they carried.

Three unique transformants were generated for each of the two 5′UTR variants. Southern analysis was used to determine whether the reporter gene cassette was incorporated into the target region of the plastid genome as well as whether the plants were homoplasmic for transformed genome. If the reporter constructs incorporate into the targeted region of the genome, EcoRI/EcoRV restriction fragments from this region will increase by 1000 bp. The Southern analysis in Fig. 2 demonstrates that the reporter constructs have incorporated into the correct region of the plastid genome. In addition, there are no fragments detected from untransformed genomes, showing that the plants are homoplasmic for the transformed genomes. Correct sequence of the promoter and 5′UTR were verified by sequencing a PCR product amplified from each transformant (data not shown).

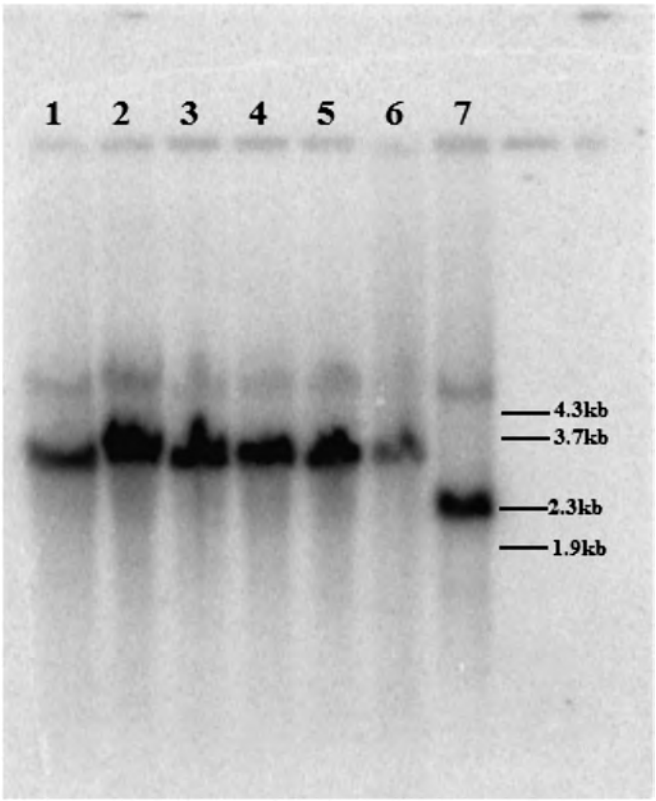


Fig. 2. Southern analysis of chloroplast transformants. Five micrograms of total leaf DNA from each unique transformant line was digested with EcoRI and EcoRV and subjected to Southern analysis using a probe complementary to *rrn16*. Lanes 1–3 are from unique transformants of the WT construct. Lanes 4–6 are from unique transformants of the Holo-sub construct. The negative control in lane 7 is from untransformed plants. The numbers to the right of the image represent migration of molecular weight standards from BstEII-digested lambda phage DNA (New England Biolabs).

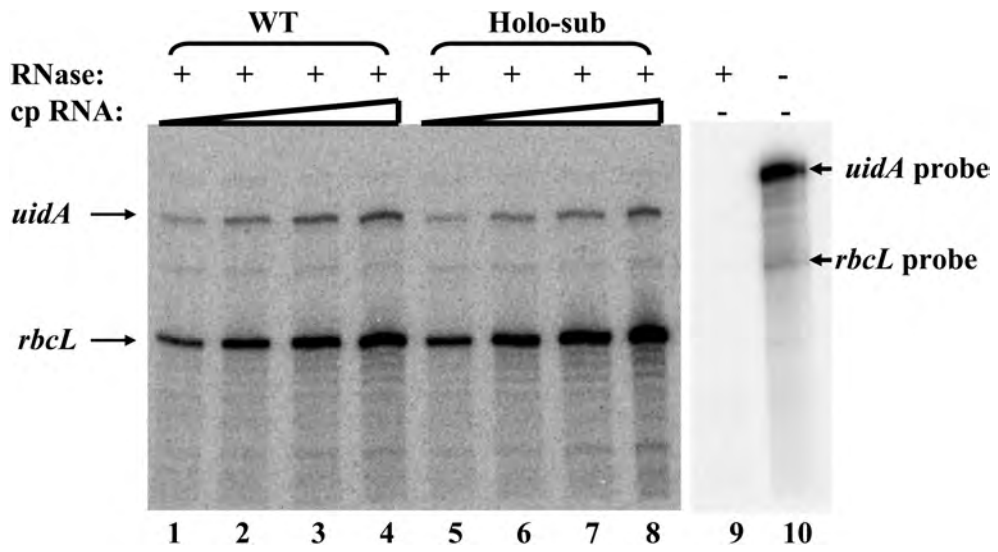


Fig. 3. Ribonuclease protection analysis of RNA from chloroplast transformants. Total chloroplast RNAs were isolated from wild-type (WT) or Holo-substitution (Holo-sub) transformants (defined in Fig. 1) and hybridized to uniformly radiolabeled RNA probes complementary to *uidA* or *rbcL*. Hybridization mixtures were subjected to digestion with single-strand-specific ribonucleases (RNase). After digestion, protected fragments were subjected to denaturing gel electrophoresis. An image from a typical experiment is shown here. Lanes 1–4 (WT) and 5–8 (Holo-sub) show results from experiments with increasing amounts (1×, 2×, 3×, 4×, respectively) of chloroplast RNA hybridized to a constant amount of probe. "*uidA*" and "*rbcL*" denote migration of the protected fragments. Lanes 9 and 10 show the results of experiments performed in the absence of chloroplast RNA, in the presence (+) or absence (-) of RNase. Migration of the undigested *uidA* and *rbcL* probes are marked. Note that the specific activity of the *rbcL* probe is substantially lower than that of *uidA* probe. This allows the visualization of protected fragments from both probes on the same image, despite the many-fold higher abundance of *rbcL* RNAs as compared to *uidA*. Although all the lanes in this figure are from the same gel, lanes 1–8 are 30-fold more exposed than lanes 9 and 10, for easier visualization.

Effects of 5′UTR on reporter RNA abundance—RPAs were used to determine RNA abundance for each transformant. In an RPA, radiolabeled probe complementary to the sequence of interest hybridizes to its target in a mixture of RNAs. After hybridization, the RNA mixture is digested with single-strand specific RNases. Probe : target hybrids survive the digestion. Protected probes are visualized and quantified after denaturing gel electrophoresis. To be certain that results accurately reflect the amount of target, probe concentrations must be in substantial excess of the target RNA. To test for probe-excess, multiple separate reactions were performed for each RPA, with varying concentrations of chloroplast RNA but a uniform concentration of probe. If the hybridization was in probe-excess, then the signal remaining after RNase digestion changed in direct proportion to the amount of chloroplast RNA in the hybridization reaction. Only data from probe-excess hybridizations were used for further analysis.

To control for pipetting or gel-loading inconsistencies, two probes were used in each assay. One was the experimental probe, which was complementary to the reporter gene *uidA*. The second was complementary to an endogenous RNA that encodes the large subunit of ribulose bis-phosphate carboxylase (*rbcL*). The quantity of *rbcL* mRNAs remains uniform in tobacco chloroplasts under the growth conditions used in these experiments (Deng and Gruissem, 1987; Rapp et al., 1992; Shiina et al., 1998). The two probes were of different lengths so that they could be easily distinguished after gel electrophoresis. As with the *uidA* probe, *rbcL* probe concentrations were also set to be in excess over the amount of target RNA. Signals from the protected *uidA* probe were normalized to that of the *rbcL* probe. An image from a typical set of RPA experiments is shown in Fig. 3.

The ratio of the uidA : rbcL RNAs was determined for each transformant, and the data from multiple assays per transformant were averaged. The chart in Fig. 4 shows the average RNA abundance for both transformants normalized to the WT transformant. ANOVA analysis of the two data sets indicates that there is no significant difference in the abundance of reporter RNA produced by either construct. Thus, we conclude that despite their substantial variation in sequence, these two 5′UTRs have the same effects on RNA abundance in vivo.

Effects of 5′UTR on reporter translation—The reporter gene in these studies is *uidA*, which encodes the *E. coli* protein β-glucuronidase, an enzyme not found in land plants (Jefferson, 1987). Because of its exceptional stability in chloroplasts, β-glucuronidase activity directly reflects the amount of *uidA* translation (Jefferson, 1987; Staub and Maliga, 1993). β-Glucuronidase activity was assayed to determine the level of translation for each transformant (Jefferson, 1987). Each of the three unique lines for each construct was assayed multiple times. The averages of the assays for each line were then averaged to obtain the translational activity for each construct.

The average translational activity for each of the two constructs, normalized to WT, is shown in Fig. 5. Translation in the Holo-sub transformants is 3% that of the WT transformants. Evaluating these results along with the RNA abundance data in Fig. 4, we see that the translation efficiency (translations per mRNA) is decreased by 33-fold for Holo-sub as compared to WT. Thus, there are sequences upstream of the putative RBS in the *atpI* 5′UTR that are required for efficient translation in vivo.

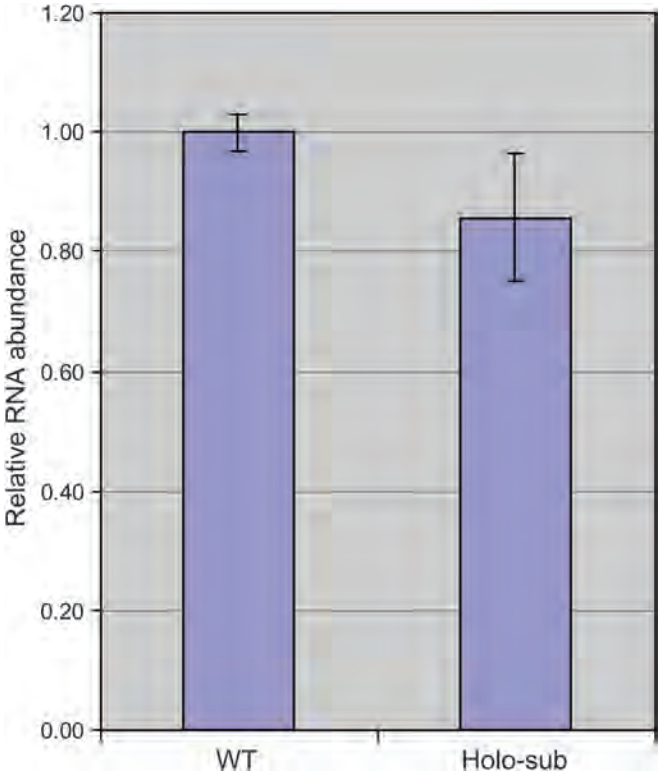


Fig. 4. Effects of 5′UTRs on RNA abundance in vivo. mRNA abundance was determined for chloroplasts transformed with reporter genes under the control of the WT or Holo-sub 5′UTR (defined in Fig. 1). All quantifications are based on ribonuclease protection assays of *uidA* RNA abundance relative to *rbcL* as the internal reference (Fig. 3). Data are from multiple assays of each unique transformant and were averaged and normalized relative to WT. Vertical bars represent ±SE. ANOVA analysis of these two data sets revealed that they are not significantly different from each other.

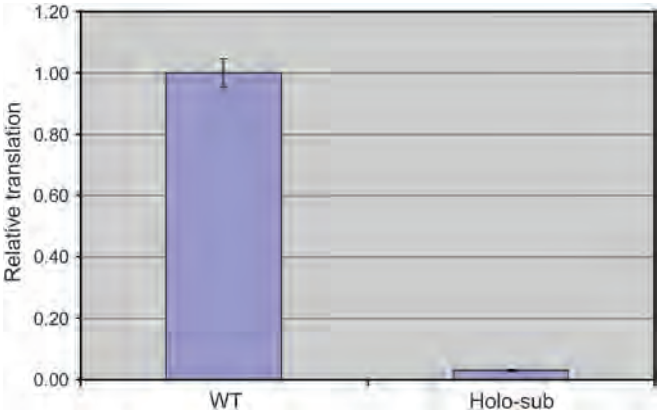


Fig. 5. Effects of 5′UTRs on translation in vivo. Average β-glucuronidase activity, which is directly proportional to *uidA* translation, was determined for WT and Holo-sub transformants (defined in Fig. 1). This chart depicts the averages normalized to WT. Vertical bars denote ±SE.

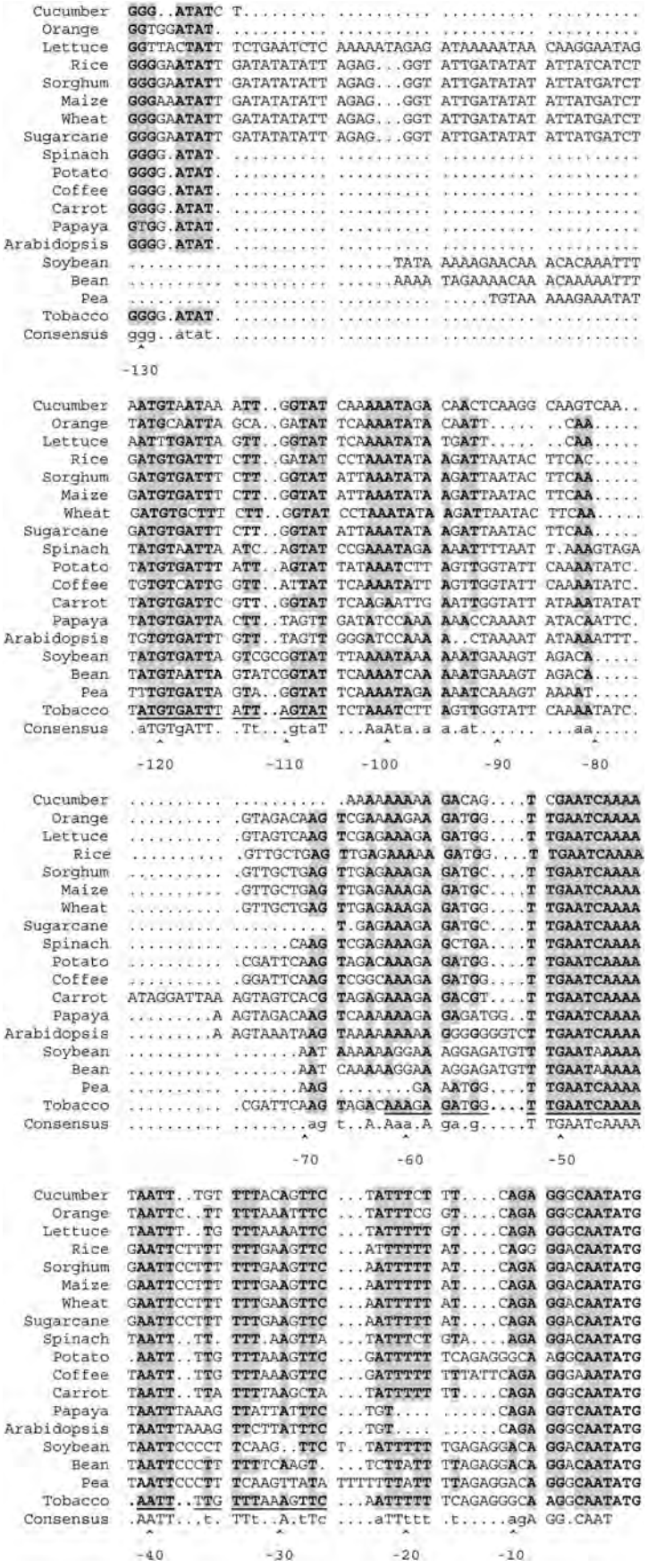
DISCUSSION

In many respects, plastids are like their prokaryotic ancestors. Their genes are arranged in a prokaryote-like fashion, in multigenic clusters rather than individually, as commonly found in eukaryotes. They contain similar polymerases and ribosomes and have at least the vestiges of most of the elements prokaryotes use to transcribe RNA and translate protein (reviewed in Sugiura et al. [1998]; Marín-Navarro et al. [2007]; Wobbe et al. [2008]). However, plastids have also evolved to include other elements that work in conjunction with these prokaryotic-like elements. In particular, plastid gene expression is controlled at the posttranscriptional level by protein factors that are encoded in the nucleus and transported into the chloroplast (Esposito et al., 2001; Nakamura et al., 2004; Asakura and Barkan, 2006; Barneche et al., 2006; Merendino et al., 2006; Raynaud et al., 2007). Regulation by nuclear-encoded factors adds a layer of complexity to chloroplast gene expression that is not found in prokaryotes.

A balance between transcription and RNA stability determines the steady state levels of mRNAs in chloroplasts (Deng and Gruissem, 1987; Green and Hollingsworth, 1992; Shiina et al., 1998; Eberhard et al., 2002; Quesada-Vargas et al., 2005). Although the majority of the RNA-encoded elements affecting RNA stability are found in the 3′UTR, some 5′UTR elements have also been shown to be important (reviewed in Schuster and Bock [2001]; Herrin and Nickelsen [2004]). For example in *Chlamydomonas*, the 5′UTR from *rbcL* has been shown to form a structure that is absolutely critical for RNA stability (Suay et al., 2005). In land plants, the *rbcL* 5′UTR has also been found to affect reporter RNA accumulation in tobacco chloroplasts in vivo (Eibl et al., 1999).

Given that both of our constructs contain identical promoters, transcription is expected to be the same in every transformant line. Therefore, any alterations in abundance of the Holo-sub transcript as compared to WT should be due to changes in RNA stability. The two 5′UTRs used in these experiments, entirely different in sequence except for the 5′-most 12 nt (derived from cloning) and the 14-nt putative RBS, caused no significant differences in the abundance of reporter RNAs. Thus if this 5′UTR has any effects on RNA stability, then the effects are likely to be mediated through the length of the 5′UTR (WT and holo-sub differed in length by only 1 nt), rather than by the primary sequence. This idea is supported by the chloroplast transformation experiments of Eibl and colleagues (1999). In those studies, RNA abundance from three constructs differing only in their 5′UTRs was positively correlated with length of the 5′UTR. Because the sequences of the RBS regions in our experiments and those of Eibl et al. differ in virtually every nucleotide, the length of the 5′UTR must have a greater effect on RNA abundance in land plants than does the RBS sequence.

In contrast to our results in tobacco, previous studies on algae have proven that there are sequence-specific regions within several *Chlamydomonas* chloroplast 5′UTRs that directly regulate mRNA stability (Monde et al., 2000; Kramzar et al., 2006). Thus, we were surprised to discover that our results support the idea that, at least for the *atpI* 5′UTR in tobacco chloroplasts, length appears to have a greater effect on RNA stability than sequence does. Perhaps this is a difference between regulation of RNA stability in algae as compared to land plants. An alternative explanation for these seemingly conflicting data are that RNA stability requires two RNA elements. The first would be a sequence- or structure-specific stabilizing element, as proven to be present in many *Chlamydomonas* chloroplast 5′UTRs (Monde et al., 2000; Kramzar et al., 2006). The second might be a sequence- or structure-specific cleavage site for the initiation


Cucumber GGG..ATATC T..... ....
Orange GGTGGATAT. ....
Lettuce GGTTACTATT TCTGAATCTC AAAAATAGAG ATAAAAATAA CAAGGAATAG
Rice GGGGAATATT GATATATATT AGAG...GGT ATTGATATAT ATTATCATCT
Sorghum GGGGAATATT GATATATATT AGAG...GGT ATTGATATAT ATTATGATCT
Maize GGGAAATATT GATATATATT AGAG...GGT ATTGATATAT ATTATGATCT
Wheat GGGGAATATT GATATATATT AGAG...GGT ATTGATATAT ATTATGATCT
Sugarcane GGGGAATATT GATATATATT AGAG...GGT ATTGATATAT ATTATGATCT
Spinach GGGG.ATAT. ....
Potato GGGG.ATAT. ....
Coffee GGGG.ATAT. ....
Carrot GGGG.ATAT. ....
Papaya GTGG.ATAT. ....
Arabidopsis GGGG.ATAT. ....
Soybean ....TATA AAAAGAACAA ACACAAATTT
Bean ....AAAA TAGAAAACAA ACAAAAATTT
Pea ....TGTAA AAAGAAATAT
Tobacco GGGG.ATAT. ....
Consensus ggg..atat. ....
-130
Cucumber AATGTAATAA ATT..GGTAT CAAAAATAGA CAACTCAAGG CAAGTCAA..
Orange TATGCAATTA GCA..GATAT TCAAAATATA CAATT..... ..CAA.....
Lettuce AATTTGATTA GTT..GGTAT TCAAAATATA TGATT..... ..CAA.....
Rice GATGTGATTT CTT..GATAT CCTAAATATA AGATTAATAC TTCAC.....
Sorghum GATGTGATTT CTT..GGTAT ATTAAATATA AGATTAATAC TTCAA.....
Maize GATGTGATTT CTT..GGTAT ATTAAATATA AGATTAATAC TTCAA.....
Wheat GATGTGCTTT CTT..GGTAT CCTAAATATA AGATTAATAC TTCAA.....
Sugarcane GATGTGATTT CTT..GGTAT ATTAAATATA AGATTAATAC TTCAA.....
Spinach TATGTAATTA ATC..AGTAT CCGAAATAGA AAATTTTAAT T.AAAGTAGA
Potato TATGTGATTT ATT..AGTAT TATAAATCTT AGTTGGTATT CAAAATATC.
Coffee TGTGTCATTG GTT..ATTAT TCAAAATATT AGTTGGTATT CAAAATATC.
Carrot TATGTGATTC GTT..GGTAT TCAAGAATTG AATTGGTATT ATAAATATAT
Papaya TATGTGATTA CTT..TAGTT GATATCCAAA AAACCAAAAT ATACAATTC.
Arabidopsis TGTGTGATTT GTT..TAGTT GGGATCCAAA A..CTAAAAT ATAAAATTT.
Soybean TATGTGATTA GTCGCGGTAT TTAAAATAAA AAATGAAAGT AGACA.....
Bean TATGTAATTA GTATCGGTAT TCAAAATCAA AAATGAAAGT AGACA.....
Pea TTTGTGATTA GTA..GGTAT TCAAAATAGA AAATCAAAGT AAAAT.....
Tobacco TATGTGATTT ATT..AGTAT TCTAAATCTT AGTTGGTATT CAAAATATC.
Consensus .aTGTgATT. .Tt...gtaT ...AaAta.a a.at..... ...aa.....
-120 -110 -100 -90 -80
Cucumber .... .AAAAAAAAA GACAG....T CGAATCAAAA
Orange ..... .GTAGACAAG TCGAAAAGAA GATGG....T TGAATCAAAA
Lettuce ..... .GTAGTCAAG TCGAGAAAGA GATGG....T TGAATCAAAA
Rice ..... .GTTGCTGAG TTGAGAAAAA GATGG....T TGAATCAAAA
Sorghum ..... .GTTGCTGAG TTGAGAAAGA GATGC....T TGAATCAAAA
Maize ..... .GTTGCTGAG TTGAGAAAGA GATGC....T TGAATCAAAA
Wheat ..... .GTTGCTGAG TTGAGAAAGA GATGG....T TGAATCAAAA
Sugarcane .... T.GAGAAAGA GATGC....T TGAATCAAAA
Spinach ....CAAG TCGAGAAAGA GCTGA....T TGAATCAAAA
Potato ..... .CGATTCAAG TAGACAAAGA GATGG....T TGAATCAAAA
Coffee ..... .GGATTCAAG TCGGCAAAGA GATGG....T TGAATCAAAA
Carrot ATAGGATTAA AGTAGTCACG TAGAGAAAGA GACGT....T TGAATCAAAA
Papaya .....A AGTAGACAAG TCAAAAAAGA GAGATGG..T TGAATCAAAA
Arabidopsis .....A AGTAAATAAG TAAAAAAAAA GGGGGGGTCT TGAATCAAAA
Soybean ....AAT AAAAAAGGAA AGGAGATGTT TGAATAAAAA
Bean ....AAT CAAAAAGGAA AGGAGATGTT TGAATAAAAA
Pea ....AAG .....GA AATGG....T TGAATCAAAA
Tobacco ..... .CGATTCAAG TAGACAAAGA GATGG....T TGAATCAAAA
Consensus ....ag t..A.Aaa.A ga.g.....T TGAATcAAAA
-70 -60 -50
Cucumber TAATT..TGT TTTACAGTTC ...TATTTCT TT....CAGA GGGCAATATG
Orange TAATTC..TT TTTAAATTTC ...TATTTCG GT....CAGA GGGCAATATG
Lettuce TAATTT..TG TTTAAAATTC ...TATTTTT GT....CAGA GGGCAATATG
Rice GAATTCTTTT TTTGAAGTTC ...ATTTTTT AT....CAGG GGACAATATG
Sorghum GAATTCCTTT TTTGAAGTTC ...AATTTTT AT....CAGA GGACAATATG
Maize GAATTCCTTT TTTGAAGTTC ...AATTTTT AT....CAGA GGACAATATG
Wheat GAATTCCTTT TTTGAAGTTC ...AATTTTT AT....CAGA GGACAATATG
Sugarcane GAATTCCTTT TTTGAAGTTC ...AATTTTT AT....CAGA GGACAATATG
Spinach TAATT..TT. TTT.AAGTTA ...TATTTCT GTA....AGA GGACAATATG
Potato .AATT..TTG TTTAAAGTTC ...GATTTTT TCAGAGGGCA AGGCAATATG
Coffee TAATT..TTG TTTAAAGTTC ...GATTTTT TTTATTCAGA GGGAAATATG
Carrot TAATT..TTA TTTTAAGCTA ...TATTTTT TT....CAGA GGGCAATATG
Papaya TAATTTAAAG TTATTATTTC ...TGT.... ......CAGA GGTCAATATG
Arabidopsis TAATTTAAAG TTCTTATTTC ...TGT.... ......CAGA GGGCAATATG
Soybean TAATTCCCCT TCAAG..TTC T..TATTTTT TGAGAGGACA GGACAATATG
Bean TAATTCCCTT TTTTCAAGT. ...TCTTATT TTAGAGGACA GGACAATATG
Pea TAATTCCCTT TCAAGTTATA TTTTTTTATT TTAGAGGACA GGGCAATATG
Tobacco .AATT..TTG TTTAAAGTTC ...AATTTTT TCAGAGGGCA AGGCAATATG
Consensus .AATT..t. TTt..A.tTc ....aTTttt .t.....agA GG.CAAT
-40 -30 -20 -10

of RNA degradation. Because the Holo-sub 5′UTR is almost entirely nonchloroplast sequence, it would be expected to have neither of those elements. Perhaps the stability of Holo-sub mRNAs is not due to length, but to the lack of a degradation signal in the 5′UTR. Further experiments are in progress to test that idea.

Plastid translation has many of the hallmarks of prokaryote translation, such as apparent Shine–Dalgarno (SD) sequences in many plastid-encoded mRNAs (reviewed in Sugiura et al. [1998]; Zerges [2000]; Marín-Navarro et al. [2007]), the conservation of sequence at the 3′ end of 16S rRNA (Maidak et al., 2001), the ribosomes' size and organization (Yamaguchi and Subramanian, 2003; Manuell et al., 2005), and similar translation initiation factors (Campos et al., 2001; Marín-Navarro et al., 2007). However, the prokaryotic model for translation initiation does not fully apply in chloroplasts. Although the SD-complementary sequence on the chloroplast 16S rRNA is highly conserved between prokaryotes and plastids (Maidak et al., 2001), the putative SD sequence is poorly conserved in chloroplasts, both in terms of primary sequence and location relative to the start codon (Hirose and Sugiura, 2004). Many genes have variably positioned SD-like sequences of unknown functionality, while several other genes contain no SD-like sequences within 200 nt upstream of the start codon (Bonham-Smith and Bourque, 1989; Hirose and Sugiura, 2004). Thus, mechanisms of translation initiation appear to have evolved in plastids since their divergence from cyanobacteria.

The RBS from different chloroplast genes have been shown to have a wide variety of effects on translation in vitro. For example, the tobacco chloroplast 5′UTRs of *rbcL* and *atpE* still contain functional ribosome binding sites with SD-like sequences, though these sequences are positioned slightly differently relative to the canonical position in prokaryotes. In contrast, a tobacco chloroplast RBS that is too close to (*petB*) or too far from (*rps12*) the start codon was found to be unnecessary for translation initiation in vitro (Hirose and Sugiura, 2004). Other experiments using tobacco chloroplast extracts have shown that an unusual GUG start codon required an extended, but correctly positioned, SD-like sequence (GAGGAGGU) for translation initiation (Kuroda et al., 2007). In vitro studies with tobacco chloroplast *psbA* found that there are two apparent ribosome binding regions in its 5′UTR. These two regions (−9 to −11 and −33 to −36) have been proposed to interact cooperatively to bind the 3′ end of the 16S rRNA, and allow for the association of a *trans*-acting protein factor(s) to a third RNA element, the AU box, during translation initiation (Hirose and Sugiura, 1996). Yet another type of RBS-mediated effect is found in the tobacco chloroplast gene *rps2*, in which the RBS acts as a negative regulator of translation in vitro (Plader and Sugiura, 2003). Translation experiments with tobacco chloroplast *atpI*, the source of the 5′UTR in this study, have not been reported, but toeprinting experiments to localize ribosome binding/pausing sites in vivo detected no ribosomes on the putative RBS of this 5′UTR in spinach chloroplasts (Stollar et al., 1994). It is clear that there is a wide variance in the utility and function of RBS sequences in chloroplasts.

In vivo experiments with several chloroplast genes in *Chlamydomonas* have mirrored in vitro studies in land plants, finding that SD-like-sequence elements were not always essential for chloroplast translation (Fargo et al., 1998; Marín-Navarro et al., 2007). In the only in vivo examination of RBS function in land plants prior to this report, the 26 nt immediately upstream of the start codon of *rbcL*, which Yukawa and colleagues had shown to contain an RBS that was functional in vitro, were placed upstream of a *uidA* reporter gene in tobacco chloroplasts (Eibl et al., 1999; Yukawa et al., 2007). Expression was compared between transformants with the truncated and full-length 5′UTRs. Reporter mRNAs with the truncated 5′UTR accumulated to a fourfold lower level than those with the full-length 5′UTR. However, the translation efficiency of the truncated 5′UTR was twofold higher than the full-length 5′UTR, resulting in only a twofold overall decrease in translation mediated by the truncated 5′UTR as compared to the wild type (Eibl et al., 1999). Thus, it appears that the region upstream of the *rbcL* RBS contains an element(s) that acts as a negative regulator of translation.

The precipitous decline in *uidA* translation in Holo-sub as compared to WT is remarkable. The most straightforward conclusion to draw from these data is that the putative RBS of *atpI* cannot support efficient translation in vivo. This conclusion is in contrast to data from Eibl et al. (1999) in which a 5′UTR consisting of only the putative RBS from *rbcL* was active in translation. The most likely explanation for these contrasting results is that the *rbcL* RBS is functional, while the putative RBS from *atpI* is not. An alternative explanation would be that the *atpI* RBS in Holo-sub is occluded by some type of structure that is not found in the WT 5′UTR. We believe that this explanation is unlikely, because the predicted free energies of computer-generated structures containing the RBS in both 5′UTRs is so low (<0.7 kcal/mole) that they would not be expected to form in vivo (data not shown; Mathews et al. [1998]).

The most likely explanation for the difference in translation for WT vs. Holo-sub is that efficient translation requires other element(s) upstream of the putative RBS of the tobacco chloroplast *atpI* 5′UTR. We hypothesize that the element(s) may be found in either or both of two highly conserved regions located upstream of the putative RBS. These two sequences, which we call Con1 and Con2, are 82% and 79%, respectively, conserved among crop plant *atpI* 5′UTRs (Fig. 6), substantially higher than the overall 57% sequence conservation of this 5′UTR. Further experiments are in progress to address the function of these highly conserved upstream elements in translation.

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Fig. 6. Alignment of *atpI* 5′UTR sequences from 18 crop plants. DNA sequences of *atpI* 5′UTRs from crop plants were aligned using Multialin software (Corpet, 1988). Capital letters in the consensus sequence denote bases present in 16 or more (>90%) of the sequences at that position. Lower case letters are present in that position in at least 12 (70–90%) of the sequences. Nucleotides matching the consensus are shaded. The numbering system is relative to the tobacco chloroplast *atpI*, where −1 is the nucleotide immediately upstream of the translation start site. Two highly conserved regions within the tobacco chloroplast sequence are underlined. The first, Con1 (bases –122 through −106), is 82% conserved relative to the other 5′UTRs in the alignment. The second, Con2 (bases −58 through −25), is 79% conserved. The ATG at the 3′ end of each 5′UTR is the translation start codon.

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